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14. ABSTRACT

The role of innate immunity in prostate cancer tumorigenesis is unclear. We hypothesis that innate immune pathways contribute to programming the inflammatory component of the tumor microenvironment and that activation of these pathways may selectively skew this immune composition and alter tumor growth. Pattern recognition receptors such as Toll-like receptors (TLRs) are key signaling molecules that regulate innate and adaptive immune responses in the presence of pathogens and endogenous ligands. We have generated and characterized TRAMP $Tg^{+/-}$ x $MyD88^{-/-}$ mice. We showed that *de novo* prostate cancers in absence of MyD88 develop higher grade adenocarcinomas than wild-type controls at 30 weeks of age. Analysis of tumor infiltrating cells revealed increased infiltration of macrophage lineage cells, characterized as myeloid-derived suppressor cells (MDSCs), and decreased CD8 T lymphocytes and NK cells. We have shown that MyD88 plays in intrinsic role in the differentiation of MDSCs, with the absence of MyD88 biasing development towards the granulocytic subtype. MyD88-deficient MDSCs have an increased migration in response to the endogenous ligand S100A9, suggesting a role of MyD88 in governing MDSC homeostasis that can be leveraged as an anti-tumor therapy.

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Introduction

Prostate cancer is the most prevalent non-skin solid malignancy and the second-leading cause of cancer-related mortality in men in the U.S.[1]. Treatment of metastatic prostate cancer with androgen-deprivation therapy ultimately leads to development of castration-resistant disease, where cancer cells become more responsive to even minute quantities of testosterone. Promising therapies are available for castration-resistant prostate cancer (CRPC), including chemotherapy, immune-based therapies, therapies targeting bone metastasis, and second line hormone therapies, however, all with a finite efficacy. Improved and likely combinatorial therapies will be necessary.

Inflammation has long been associated with the prostate cancer microenvironment, and may facilitate tumor growth or promote an anti-tumor immune response. Evidence suggests that cancer cells can be hijacking inflammatory pathways to promote angiogenesis and proliferation[2]. Conversely, inflammation can trigger the infiltration of cytotoxic immune effector cells, resulting in the production of clonal CD8^{+} T cells[3]. However, the contribution of the tumor infiltrating lymphocytes (TILs) to prostate cancer development, growth, and metastasis is unclear. We are interested in understanding the mechanisms for development of TILs and how they modulate prostate cancer. Our hypothesis is that the innate immune response can program TILs and play a key role in tumor surveillance, are important in generation of tumor-specific immunity, and that by tumor growth can be altered through modulating the composition of TILs through innate immunity.

Body

Pathogens or cancerous cells alike can produce danger signals that elicit the activation of immune responses. These signals in the form of conserved molecules termed pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) can be discriminated from self-antigens by a family of pattern-recognition receptors of innate immunity, including Toll-like receptors (TLRs). Thirteen mammalian TLRs have been identified to date with ligands ranging from lipopolysaccharide (LPS) found in gram-negative bacterial walls recognized by TLR4, double stranded RNA produced by many viruses for TLR3, viral CpG motifs with TLR9, to endogenous ligands such as heat-shock protein 70 and chromatin component HMG-B1. Activation of these receptors leads to induction of multiple inflammatory pathways, including nuclear factor-kappa B (NF-κB) and interferon regulatory factors (IRFs), which may mediate the development of cytotoxic T lymphocytes (CTLs) and dendritic cell (DC) maturation[4]. Although TLRs have been shown to inhibit negative regulatory cells such as Tregs, the relationship between TLRs and myeloid-derived suppressor cells (MDSCs) is less clear[4-5].

TLRs recruit adaptor proteins such as MyD88 and serine kinase IL-1 receptor-associated kinase (IRAK), leading to activation of MAP kinases, NF-κB, and expression of inflammatory genes. Most TLRs utilize the MyD88 pathway. The role of TLRs in modulating cancer is conflicting, as prior reports have suggested tumor promoting as

well as suppressing effects. Deficiency in MyD88 confers decreased development of tumors in a mouse model of spontaneous intestinal tumorigenesis and diethylnitrosamine-induced hepatocellular tumors[6-7]. In contrast, a recent report suggested that MyD88 inhibition promoted pancreatic cancer growth through dendritic cell and Th2 activation[8].

We have been focused on studying the phenotype of TRAMP Tg$^{+/-}$ x MyD88$^{-/-}$ mice, work described in **Specific Aim 2**. This has culminated in a publication now e-published and in the April 2015 Edition of *The Prostate,* entitled "Loss of MyD88 Leads to More Aggressive TRAMP Prostate Cancer and Influences Tumor Infiltrating Lymphocytes". In summary, we showed that the absence of MyD88 led to increased prostatic intraepithelial neoplasm (PIN) and areas of well-differentiated adenocarcinoma in TRAMP transgenic mice. Analysis of infiltrating immune populations revealed an increase in CD11b$^+$ cells and a deficiency in NK cells in prostates from MyD88$^{-/-}$ TRAMP$^{Tg+/-}$ compared to MyD88$^{+/+}$ TRAMP$^{Tg+/-}$ mice, whereas a decrease in splenocytic NK cell differentiation was observed in MyD88$^{-/-}$ mice. Prostate tumors revealed no significant differences in NF-κB or AR expression in MyD88$^{+/+}$ TRAMP$^{Tg+/-}$ compared to MyD88$^{-/-}$ TRAMP$^{Tg+/-}$ mice.

In our 2014 Annual Summary, we presented limitations to our initial aims using TRAMP Tg$^{+/-}$ animals, namely the length of time for development of tumors from 24 to 30 weeks of age, the ubiquitous presence of our gene knockout in prostate epithelium, stroma, as well as immune system, and the fixed nature of the prostate model with expression of the large T antigen, which may have limited translational implications. We proposed a model of disease progression in prostate cancer, where damage-associated molecular patterns (DAMPs) released by the tumor stimulate the innate immune pathways through pattern recognition receptors (PRRs) including the TLRs and intracellular Nod-like receptors (NLRs). To parse out the role of TLR signaling in various compartments, we proposed adapting a previously published subcutaneous prostate tumor model based on lentiviral transfection of primary prostate epithelium[11-12]. This model has been developed in Owen Witte's laboratory at UCLA, which we are collaborating with. Prior reports have been performed using both human and murine prostate epithelium on an immunocompromised background. We tested a syngenic immunocompetent model using murine prostate epithelium on a C57Bl6 host which led to exciting preliminary data showing that just the presence of an intact immune system altered tumor growth with larger tumors in a C57Bl6 versus SCID background (**Fig 1**). The flexibility of the model allows variation in the oncogenic drivers of the tumors, which subsequently produces disease ranging from PIN (AKT/ERG and TRAMP) to castration-resistant prostate cancer (AKT/ERG + AR). As the tumor cells are combined with fetal mesenchymal cells for implantation, this model allows for the genotypic manipulation of both the tumor and its surrounding stroma. We presented preliminary data using this system in our 2015 Annual Summary. However, over the past year, we encountered complications with poor lentiviral infection efficiency and expression of AKT. During this time, we continued studying the mechanisms of increased CD11b$^+$

cells in MyD88$^{-/-}$ TRAMP$^{Tg+/-}$ compared to MyD88$^{+/+}$ TRAMP$^{Tg+/-}$ mice, which we present in this 2016 Annual Summary below.
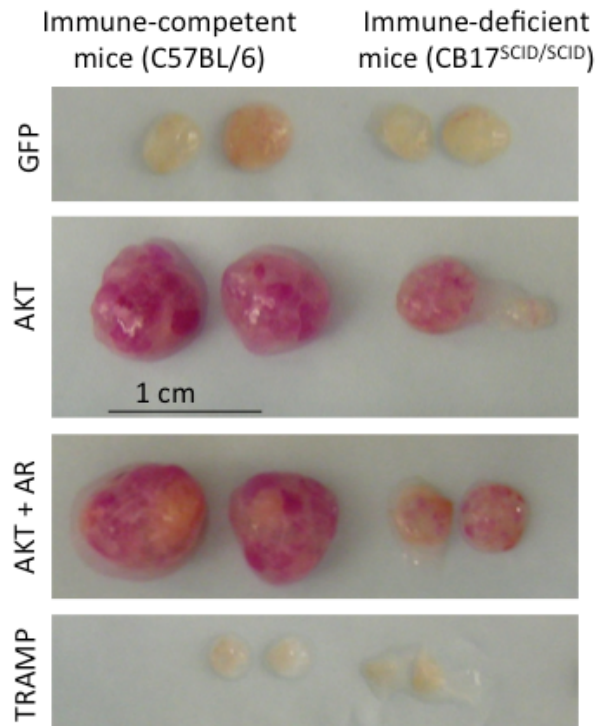

Figure 1. Larger C57Bl6 tumors generated by retroviral infection by AKT and AKT + AR in a C57Bl6 versus CB17scid/scid background.

Recently, we performed further staining of infiltrating immune populations in tumors from 30 week-old MyD88$^{+/+}$ TRAMP$^{Tg+/-}$ and MyD88$^{-/-}$ TRAMP$^{Tg+/-}$ mice, which revealed an increase in CD11b^{+}Gr1^{+} cells suggestive of myeloid-derived suppressor cells (MDSCs). Analysis of tumors by qPCR revealed an increase in iNOS and l-arginine, which mediate the T cell inhibitory function of MDSCs. However, we found no significant differences in cytokines and chemokines important in MDSC development and recruitment such as IFNγ, and chemotactic molecules such as HMG-B1 and SA100A9. To further examine the potential mechanisms and to determine if there is an intrinsic role of MyD88 to develop myeloid subtypes, we optimized and performed *in vitro* differentiation of bone marrow to various myeloid subtypes using combinations of GM-CSF, G-CSF, and M-CSF (**Fig. 2**). We found that MyD88-dependent signaling pathways are important in the homeostasis of T cell inhibitory granulocytic MDSCs as MyD88$^{-/-}$ bone marrow preferentially developed CD11b^{+}Ly6G^{hi}Ly6C^{lo} granulocytic MDSCs compared to CD11b^{+}Ly6G^{lo}Ly6C^{hi} monocytic MDSCs. Ongoing experiments are characterizing the *in vitro* response of differentiated MDSC subpopulations to functional assays. Using a transwell migration assay, we have shown that bone marrow derived MyD88$^{-/-}$ CD11b^{+}Ly6G^{hi}Ly6C^{lo} granulocytic MDSCs have increased migration in response to the endogenous inflammatory protein SA100A9, but not HMGB1 and exogenous ligands polyIC and CpG compared to MyD88$^{+/+}$ CD11b^{+}Ly6G^{hi}Ly6C^{lo} granulocytic MDSCs (**Fig. 3**).

This work is building on our ongoing model suggesting that MyD88 functions as a negative regulator of MDSC differentiation to the granulocytic subtype and promoting anti-tumor immunity. We propose that activation of TLR signaling through MyD88-dependent pathways may induce an anti-tumor program through suppression of negative T cell regulators by inhibiting MDSC development and migration, and may be an adjunct in the treatment of prostate cancer.
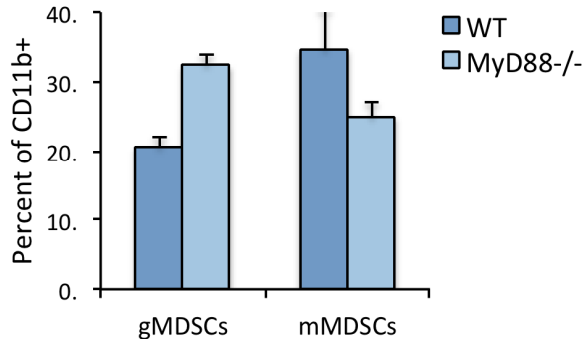
Figure 2. Increased granulocytic CD11b$^+$Ly6G^{hi}Ly6C^{lo} MDSCs from *in vitro* differentiated bone marrow derived from MyD88$^{-/-}$ compared to MyD88$^{+/+}$ mice.
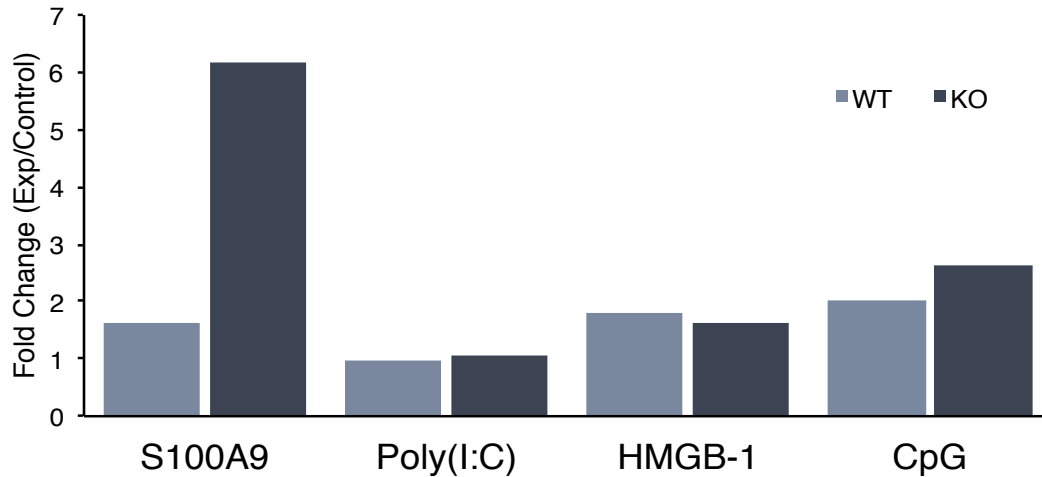
Figure 3. Increased migration of CD11b$^+$Ly6G^{hi}Ly6C^{lo} MDSCs in response to S100A9 from *in vitro* differentiated bone marrow derived from MyD88$^{-/-}$ compared to MyD88$^{+/+}$ mice.

Key Research Accomplishments

- We have published our manuscript describing the role of MyD88 in prostate cancer tumorigenesis and composition of the immune microenvironment in *The Prostate*
- We have shown an intrinsic increase in granulocytic CD11b$^+$Ly6G^{hi}Ly6C^{lo} MDSC development from bone marrow-derived *in vitro* differentiated cells
- We have shown that MyD88$^{-/-}$ granulocytic CD11b$^+$Ly6G^{hi}Ly6C^{lo} MDSCs have increased migration in response to stimuli by S100A9, but not HMGB1, CpG, and polyIC compared to the wild-type counterpart.

Reportable Outcomes

We have presented this work in yearly seminars at the UCLA Prostate SPORE Lecture Series as well as the manuscript published in *The Prostate.*

Conclusions

We have shown that TRAMP Tg$^{+/-}$ x MyD88-deficient mice result in accelerated prostate cancer development with increased infiltration of immature CD11b$^+$Gr1$^+$ myeloid cells and decreased T lymphocytes. We have shown a MyD88-dependent intrinsic ability to skew the differentiation between granulocytic and monocytic MDSCs and to migration in response to S100A9. We propose that activation of TLR signaling through MyD88-dependent pathways may induce an anti-tumor program through suppression of negative T cell regulators by inhibiting MDSC development and migration, and may be an adjunct in the treatment of prostate cancer.

References

1. Siegel, R., Naishadham, D. & Jemal, A. Cancer statistics, 2012. *CA: a cancer journal for clinicians* **62,** 10–29

2. Brigati, C., Noonan, D. M., Albini, A. & Benelli, R. Tumors and inflammatory infiltrates: friends or foes? *Clinical & experimental metastasis* **19,** 247–58 (2002).

3. Sfanos, K. S. *et al.* Human prostate-infiltrating CD8+ T lymphocytes are oligoclonal and PD-1+. *The Prostate* **69,** 1694–703 (2009).

4. Modlin, R. L. & Cheng, G. From plankton to pathogen recognition. Nat Med 10, 1173-4 (2004).

5. Ostrand-Rosenberg, S. & Sinha, P. Myeloid-derived suppressor cells: linking inflammation and cancer. J Immunol 182, 4499-506 (2009).

6. Rakoff-Nahoum, S, Medzhitov, R. Regulation of spontaneous intestinal tumorigenesis through the adaptor protein MyD88. Science 317(5834), 124-127 (2007).

7. Naugler, W.E., Sakurai, T., Kim, S., Maeda, S., Kim, K., Elsharkawy, A.M., Karin, M. Gender disparity in liver cancer due to sex differences in MyD88-dependent IL-6 production. Science 317(5834), 121-4 (2007).

8. Ochi, A., et al., MyD88 inhibition amplifies dendritic cell capacity to promote pancreatic carcinogenesis via Th2 cells. Journal of Experimental Medicine 209(9), 1671-7 (2012).

9. Gannon et al., Large scale independent validation of the nuclear factor-kappa B p65 prognostic biomarker in prostate cancer. Eur J Cancer 13 (2013).

10. Gannot, G., et al., Decrease in CD8+ lymphocyte number and altered cytokine profile in human prostate cancer. Am J Cancer Res, 1(1) 120-7, (2011).

11. Goldstein, A.S., Huang, J., Guo, C., Garraway, I.P., Witte, O.N. Identification of a cell of origin for human prostate cancer. Science 329(5991), 568-71 (2010).

12. Goldstein, A. S. *et al.* Purification and direct transformation of epithelial progenitor cells from primary human prostate. *Nature protocols* **6,** 656–67 (2011).